Effect of Sugar and Low-Dose Irradiation on Toxin Production by *Clostridium botulinum* in Comminuted Bacon

ABSTRACT

Comminuted bacon, processed to contain target levels of 40 μg $NaNO_2$/g and 0, 0.25 or 0.75% sucrose or 0.75% glucose, was inoculated with a mixture of spores of 20 strains of *Clostridium botulinum* (400 spores per g) and was canned under vacuum. Portions were irradiated using ^{137}Cs at doses of 0, 0.19, 0.38, 0.75, 1.12 and 1.5 Mrad. Cans were incubated for 1, 2, 4 or 8 wk at 30°C. Some cans of nonirradiated bacon without or with 0.25% sucrose became toxic in 2 wk; with 0.75% sucrose, toxin production was delayed to 8 wk. Bacon irradiated at 0.75 Mrad, made with or without sucrose, became toxic in 2 to 4 wk, whereas most cans of bacon irradiated at 1.5 Mrad remained toxin-free for the 8-wk incubation period. A comparison of bacon made with 0.75% sucrose or glucose showed no difference between the sugars in the rates of toxin production by *C. botulinum* in irradiated cans of bacon. Irradiation at 0.19 Mrad increased the rate of toxin formation over nonirradiated bacon in sugar-containing (0.75%) bacon, but had no effect in sugar-free bacon. The pH of nonirradiated bacon containing 0.75% glucose or sucrose decreased from pH 6.12 and 6.11, respectively, to pH 5.63 and 5.67 after 8 wk of incubation at 30°C. The titratable acidity showed a concurrent increase. The pH and titratable acidity of bacon irradiated at 0.19 Mrad or higher showed no changes.

A recent review (*1*) described some applications of ionizing irradiation that have been approved for food use by some countries. Although high dose irradiation (1 Mrad or more) has been approved in The Netherlands for spice radicidation (sterilization) since 1971, most approved uses have been for low dose (<1 Mrad) applications.

Concern has been expressed that low dose irradiation may selectively destroy radiation-sensitive harmless bacteria, allowing the proliferation of radiation-resistant food pathogens (*1,14*). *Clostridium botulinum* is of particular interest because its spores have relatively large irradiation D-values ranging from 129 to 334 Krad (*3*). Irradiation doses of 1 Mrad or less are not sufficient to guarantee destruction of these spores; doses of 1.0 to 1.5 Mrad are required to effect a 6-$\log_{10}$ reduction (*10*), and doses as high as 4.5 Mrad are required to effect sterility equivalent to that commercially obtained from thermal processing (*2,12*). An area of potential concern is that low dose irradiation could cause selective destruction of acid-producing microorganisms normally present in semipreserved meat products (*9*), permitting more rapid growth of *C. botulinum*. Conversely, it is possible that low dose irradiation could selectively destroy oxygen-consuming organisms, delaying *C. botulinum* growth due to higher oxidation-reduction potentials.

The purpose of our studies was to determine whether low dose gamma irradiation in low nitrite (40 μg/g) bacon altered the rate of *C. botulinum* toxin formation. Because acid-producing microorgansims, such as *Streptococcus, Leuconostoc, Pediococcus* and *Lactobacillus* are often found in bacon (*5*), we also wanted to determine whether acid production was influenced by the sugar content of bacon.

MATERIALS AND METHODS

Bacon preparation

Hog bellies weighing between 3.85 and 5.80 kg were obtained from a local processing plant. These were hand pumped to 111% of green weight using a brine consisting of 15% NaCl, 3% sodium tripolyphosphate, 0.55% sodium isoascorbate and 0.040% sodium nitrite. Sugar (sucrose or glucose) was added to some brines at concentrations of 2.5 or 7.5%. The drained weight (overnight at 4°C) averaged 109.9% of green weight. The pumped and drained bellies were processed in a smokehouse with natural smoke to an internal temperature of 55.9°C. The processed and chilled bacon bellies averaged 101.7% of green weight. Two bellies were processed for each experimental variable. The processed bacon was tempered in a freezer until firm (to about -20°C) and was then ground through a 3/16-in. (4.8-mm) plate or chopped in a bowl cutter. The bacon was then mixed in a Hobart mixer and 2-kg portions were placed in 30 × 38-cm polyethylene pouches which were

then heat-sealed under vacuum. These were kept frozen until needed. Packages were thawed by placing in warm water (40°C) with frequent kneading.

Clostridium botulinum *spores*

Twenty proteolytic strains of *C. botulinum* were used (Table 1). Spore crops were prepared by growing cultures in Trypticase soy broth (BBL Microbiology Systems) containing 0.1% sodium thioglycollate; incubation was for 1 wk at 33°C in an anaerobic incubator (National Appliance Company) under N_2. Cultures were centrifuged at 3,000 × *g* for 15 min and washed once with distilled water. The pellet was resuspended in water to one-tenth of the original volume; the suspensions were then heated at 80°C for 10 min. The spores were enumerated by diluting in freshly autoclaved distilled water and plating using a two-layer technique. The inoculum was mixed with the bottom layer (10 to 12 ml of Brewer anaerobic agar) which after solidification was overlaid with 12 to 15 ml of the same agar. The plates were immediately placed in Brewer anaerobic jars (BBL) which were evacuated to 20 in. Hg vacuum. The vacuum was replaced with a mixture of 5% H_2, 10% CO_2, and 85% N_2. The evacuation and replacement was repeated twice (total of three times); the anaerobic jars were incubated at 35°C for 2 d.

A spore mixture was made from the twenty suspensions. Equal quantities of spores were combined to give a final concentration of 2.8×10^5 spores/ml. The spore mixture was stored in 15-ml serum bottles at 4°C until needed.

Bacon inoculation

Thawed bacon was spread out in a layer about 1.5-cm thick. The spore suspension, at a rate of 1 ml for each 700 g of comminuted bacon (final concentration 400 spores/g), was pipetted dropwise onto the surface and was evenly distributed with gloved hands. The inoculated bacon was distributed into 208 × 107 aluminum tab cans, approx. 70 g per can. The cans were sealed in a Rooney vacuum canner (18 to 21 in. Hg vacuum).

Irradiation

The bacon cans were placed in #10 size metal cans to which were added crushed ice and water for temperature control. Irradiation was at a rate of 10.93 Krad/min using a ^{137}Cs source; the doses used were 0, 0.19, 0.38, 0.75, and 1.12 and 1.50 Mrad.

Incubation

Twenty cans were incubated at 30°C for periods up to 8 wk. Five cans were removed for toxin analysis at 1, 2, 4 or 8 wk, or earlier if swelling occurred before these times.

Toxin testing

Extracts of cans' contents were prepared by adding 20 ml of gelatin phosphate buffer (0.2% gelatin, 0.36% KH_2PO_4, 0.15% $Na_2HPO_4 \cdot 7H_2O$, pH 6.2) to 10 g of the bacon in 50-ml centrifuge tubes. After mixing with a wooden tongue depressor, the tubes were centrifuged at 3,000 × *g* for 15 min at 4°C. Pairs of Swiss-Webster mice (15 to 20 g) were injected i.p. with 0.5 ml of the extracts. The mice were observed for typical respiratory symptoms of botulism; if these occurred or if the mice died, the extracts were boiled 10 min and mice were again injected. Extracts were considered positive if the boiled preparations failed to kill the mice or elicit the symptoms.

Measurement of pH and titratable acidity

Uninoculated cans of bacon were incubated in a manner similar to that described above. After the prescribed incubation intervals, 50 g of the comminuted bacon was placed in a Stomacher bag with 100 ml of distilled water. After 2 min of blending, the homogenates were filtered through two layers of cheesecloth and 30-ml portions were removed for pH and titratable acidity measurements using a combination electrode. Titratable acidity was determined by adding enough 0.10 N NaOH to bring the pH to 7.0.

Most probable number counts

Spore counts in bacon before incubation were made by adding in triplicate 20-g portions to 180 ml of sterile water containing 0.1% sodium thioglycollate. These were blended for 10 s in a blender. Serial $\log_{10}$ dilutions were made by adding 1 ml to 9 ml of freshly autoclaved fluid thioglycollate medium. The tubes were incubated in an anaerobic incubator at 33°C under N_2. Tubes showing turbidity were tested for toxin by centrifuging and injecting the supernatant fluids into mice.

TABLE 1. C. botulinum *strains*.

No.	Origin	Type	No.	Origin	Type
3	FDA[a]	A	4	APHIS[f]	B
78	FDA	A	770B	APHIS	B
429	FDA	A	7949	ATCC	B
62	FDA	A	169	FDA	B
69	FDA	A	383	FDA	B
426	FDA	A	999	FDA	B
33	Natick[b]	A	8688R	FDA	B
B1218	NRRL[c]	A	642	FDA	B
20PLALC	CDC[d]	A	53	Natick	B
25763	ATCC[e]	A	17409-1A	CDC	B

[a]FDA, Food and Drug Administration, Washington, DC.
[b]Natick, U.S. Army Natick Research and Development Center.
[c]NRRL, Northern Regional Research Laboratories, U.S. Department of Agriculture, Peoria, IL
[d]CDC, Centers for Disease Control, Atlanta, GA.
[e]ATCC, American Type Culture Collection, Rockville, MD.
[f]APHIS, Animal and Plant Health Inspection Service (now Food Safety and Inspection Service), U.S. Department of Agriculture, Beltsville, MD.

Moisture and NaCl determination

Moisture was determined by an Association of Analytical Chemists' method (*4*). NaCl was determined by resuspending an ashed (550°C) sample in distilled water and determining absorbance of the Na using a Perkin-Elmer atomic absorption spectrophotometer. Dried NaCl (160°C, 4 h) was used to prepare Na standards.

RESULTS AND DISCUSSION

Two separate experiments were done. In the first, three lots of bacon were prepared with target levels of 0, 0.25 and 0.75% sucrose. The actual sucrose concentrations, however, based on calculations of retained brine after the bacon was processed, were 0.23 and 0.72%. MPN determination of spores in the three bacons of the first experiment were 200, 290 and 400 spores per g, respectively. In the second experiment, comparing sucrose and glucose at target levels of 0.75%, the calculated concentrations of the two sugars were 0.74%; the spore MPN counts for the bacon were 240 (control), 240 (sucrose) and 400 (glucose). These MPN values were within the 95% confidence interval for 400 spores per g; for levels of 0.1, 0.01 and 0.001 g, the MPN of 460 spores per g, for example, encompasses the range of 71 to 2400 spores per g.

Results of the first experiment comparing two sucrose levels are presented in Table 2. Nonirradiated bacon or that made to contain 0.25% sucrose became toxic at the second week; nonirradiated bacon with 0.75% sucrose, however, remained free of toxin for 4 wk, although by the 8th week all cans (5/5) tested were toxic. Irradiation at 0.75 Mrad gave the same results (toxin in 2 wk) in the bacon made without sucrose or with 0.25% sucrose. Bacon irradiated at 0.75 Mrad containing the higher level of sucrose (0.75%), however, became toxic as rapidly as the control bacon or that formulated to contain 0.25% sucrose. This indicated that irradiation at 0.75 Mrad was insufficient to inactivate the spores of *C. botulinum*. When bacon was irradiated at 1.5 Mrad, 2/60 cans tested showed toxin. This was not surprising because Anellis and Koch (*3*) reported that 10 strains of *C. botulinum* spores (types A and B) had an average irradiation D-value of approx. 0.30 Mrad. Assuming a similar D-value for the 20 strains used in this experiment, a starting spore concentration of 400 per g would be reduced to 0.004 per g or 0.28 spores per can. Some cans, as in this experiment, might therefore contain viable spores which could germinate, outgrow and produce toxin. An irradiation dose of 0.75 Mrad, on the other hand, would reduce the spore population only by 2.5-$\log_{10}$ cycles, hence most cans should contain viable spores.

Results of this experiment, showing that the rate of toxin production by *C. botulinum* decreased in nonirradiated bacon containing the higher (0.75%) level of sucrose, suggest that the inhibition might have been caused by the growth of acid-producing bacteria as shown by Reiman et al. (*9*) when 1% glucose was added to a variety of semipreserved meat products.

The hypothesis of increased acid production by indigenous bacteria was explored further in a second experiment where either glucose or sucrose at a target level of 0.75% was incorporated with the bacon. Results of the toxin analyses are shown in Table 3. Cans of bacon made without sugar and not irradiated were toxic by 1 wk (2/5 cans) and all except four cans were toxic by the 4th week. The four nontoxic cans had been removed before the specified incubation period due to swelling. When irradiated at 0.19 Mrad, all tested cans were toxic in 1 wk, and by 2 wk all had swollen and were toxic. Irradiation at higher doses progressively delayed the rate of toxin production in the sugar-free bacon; at 1.12 Mrad, only 2/20 cans were toxic at 8 wk.

When bacon was formulated with 0.75% glucose or sucrose (Table 3), there were no toxic cans until the second week in the nonirradiated controls. At this time, 2/5 cans of glucose-containing bacon and 1/5 cans of sucrose-containing bacon were toxic; all were toxic by 2 wk. All bacon cans irradiated at 0.19 Mrad became toxic at 2 wk, indicating possible destruction of indigenous bacteria inhibitory to *C. botulinum*. At an irradiation dose of 0.38 Mrad, cans remained toxin-free for 1 wk, but all cans were toxic at the second week. Irradiation at 1.12 Mrad was sufficient to inactivate most of the spores, i.e., spores were unable to germinate, outgrow and produce

TABLE 2. *Effect of sucrose and irradiation on toxin formation by* C. botulinum *in bacon*[a].

Sucrose (%)	Irradiation (Mrad)	No. of toxic cans[b] 1 wk	2 wk	4 wk	8 wk	Total toxic
0	0	0/5	4/5	10/10 (9)[c]	-	14/20
0.25	0	0/5	4/5	10/10 (10)	-	14/20
0.75	0	0/5	0/5	0/5	5/5 (5)	5/20
0	0.75	0/5	5/5	10/10 (10)	-	15/20
0.25	0.75	0/5	5/5	10/10 (10)	-	15/20
0.75	0.75	0/5	3/5	10/10 (10)	-	13/20
0	1.50	0/5	0/5	0/5	0/5	0/20
0.25	1.50	0/5	0/5	2/5	0/5	2/20
0.75	1.50	0/5	0/5	0/5	0/5	0/20

[a]Bacon contained 40 mg $NaNO_2$/kg.

[b]Five cans removed at each interval except swollen cans which were removed when swelling occurred.

[c]Numbers in parentheses indicate number of swollen cans.

toxin; only 2/60 cans from all treatments were toxic at 8 wk.

The data in Tables 2 and 3 on toxin formation and numbers of swollen cans confirm the observation that toxin production precedes can swelling (*8*). In the first experiment (Table 2), none of the cans was visibly swollen by the second week, although most of the nonirradiated and 0.75 Mrad-irradiated cans were toxic. Between 2 and 4 wk, enough time had elapsed so that all but three toxic cans were also swollen. In the second experiment (Table 3), toxicity developed earlier but many toxic cans had not become swollen.

Evidence that acid production occurred in bacon with sugar is presented in Table 4. Control bacon made without sugar showed no change in pH or titratable acidity regardless of irradiation dose or storage time. Nonirradiated bacon made with 0.75% glucose or sucrose, however, showed a decrease in pH in 1 wk from pH 6.33 (control bacon) to 6.11 (sucrose) and 6.12 (glucose). At 8 wk, these pH values were, respectively, pH 6.28, 5.67 and 5.63. Irradiation at a dose of 0.19 Mrad or higher, however, inhibited acid production indicating that acid-producing bacteria were inactivated by this dose.

The bacon used in our studies contained 5.45% (moisture phase) NaCl. Roberts and Ingram (*11*) found that types A and B *C. botulinum* were inhibited by 5.0% NaCl (wt/vol) at pH 6.2, but only if $NaNO_2$ (50 μg/g) was included in the culture medium. Both types were in-

TABLE 3. *Effect of glucose and sucrose on* C. botulinum *toxin production in irradiated bacon*[a].

Sugar (0.75%)	Irradiation (Mrad)	No. toxic cans[b] 1 wk	2 wk	4 wk	8 wk	Total
0	0	2/5	4/5	10/10 (7)[c]	-	16/20
0	0.19	5/5	15/15 (15)	-	-	20/20/
0	0.38	0/5	15/15 (15)	-	-	15/20
0	0.75	0/5	1/5	8/10 (10)	-	9/20
0	1.12	0/5	0/5	0/5	2/5 (2)	2/20
Glucose	0	0/5	2/5	5/5 (1)	5/5	12/20
Glucose	0.19	2/5	13/13 (13)	2/2 (2)	-	17/20
Glucose	0.38	0/5	14/15 (15)	-	-	14/20
Glucose	0.75	0/5	2/5	7/10 (8)	-	9/20
Glucose	1.12	0/5	0/5	0/5	0/5 (1)	0/20
Sucrose	0	0/5	1/5	5/5	5/5 (5)	11/20
Sucrose	0.19	1/5	5/5	10/10 (10)	-	16/20
Sucrose	0.38	0/5	5/5	10/10 (10)	-	15/20
Sucrose	0.75	0/5	0/5	4/5	2/5 (3)	6/20
Sucrose	1.12	0/5	0/5	0/5	0/5	0/20

[a]Bacon was made with a target level of 40 mg $NaNO_2$/kg.
[b]Twenty cans per treatment; five cans were removed at each interval except swollen cans which were removed when swelling occurred.
[c]Numbers of cans showing swelling are in parentheses.

TABLE 4. *Effect of glucose and sucrose on pH and titratable acid of comminuted bacon.*

Sugar (0.75%)	Irradiation (Mrad)	pH 1[b]	2	8	Titratable acidity (%) 1	2	8
0	0	6.33	6.30	6.28	4.53	4.75	5.33
0	0.19	6.37	6.36	6.33	4.45	4.38	4.66
0	0.38	6.36	6.37	6.30	4.42	4.56	4.92
0	0.75	6.39	6.33	6.36	4.35	4.63	4.24
0	1.12	6.33	6.35	6.37	4.62	4.59	4.49
Glucose	0	6.12	5.93	5.63	5.68	6.97	8.26
Glucose	0.19	6.32	6.37	6.33	4.41	4.27	4.71
Glucose	0.38	6.38	6.37	6.30	4.18	4.36	4.71
Glucose	0.75	6.36	6.36	6.35	4.44	4.63	4.67
Glucose	1.12	6.38	6.39	6.35	4.29	4.22	4.78
Sucrose	0	6.11	5.93	5.67	5.47	6.22	8.07
Sucrose	0.19	6.35	6.40	6.35	4.06	4.05	4.28
Sucrose	0.38	6.38	6.38	6.30	4.23	4.05	4.30
Sucrose	0.75	6.37	6.40	6.35	3.78	4.06	4.32
Sucrose	1.12	6.40	6.36	6.35	3.96	3.73	4.04

[a]Amount of 0.10 N NaOH required to bring pH to 7.0 (see text).
[b]Weeks of incubation at 30°C (uninoculated cans).

hibited by 6% NaCl provided that the pH was decreased to pH 5.8 to 6.0. At a pH of 5.6 and 5% NaCl, there was little or no inhibition unless 50 μg $NaNO_2$/g was added. Our results on nonirradiated sugar-containing bacon are in general agreement with the results of Roberts and Ingram (*11*).

The indigenous acid-producing bacteria of meat have long been used as a means of producing certain products of sufficient acidity to prevent growth of pathogenic microorganisms. However, they cannot always be relied on to produce sufficient acid for this purpose (*9*). The current practice is to inoculate meat products with cultures of *Pediococcus acidolactici* or *Lactobacillus plantarum* to insure sufficient acid production. Tanaka et al. (*13*) showed that acid production (pH 5.8) occurred in sucrose-containing (0.9%) bacon in uninoculated samples, but supplementation with *L. plantarum* (4×10^6 per g) provided greater and more consistent acid production (pH 4.4). This suggested that the indigenous bacteria were not as effective as *L. plantarum* in acid production. Our results (lowest pH 5.63) indicate that indigenous bacteria, though capable of producing considerable acid, could not produce enough to completely inhibit *C. botulinum*, for this, a pH of 4.6 or lower is required (*6,7*).

Our results indicate that indigenous acid-producing bacteria delay toxin formation by *C. botulinum* in low-nitrite comminuted bacon, but only at a sugar concentration of 0.75%. Low-dose irradiation (0.19 Mrad) prevented acid production in the bacon probably by inactivating the acid-producing bacteria and as a result, more rapid toxin production of *C. botulinum* occurred. Hence, it is possible that low dose irradiation (0.19 Mrad) might eliminate the inhibitory effect of acid-producing microorganisms in products containing relatively high levels of fermentable sugar.

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